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Analysis of synovial fluid based upon native fluorescence spectroscopy

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Abstract : The present study is aimed to address the applications and development of native fluorescence spectroscopy as a diagnostic tool and to characterize the synovial fluid of normal and rheumatic disease. To quantify the spectral differences and to classify the fluids, an intensity ratio parameter $R_1 = I_{(323)}/I_{(393)}$ is introduced and the area under the curve analysis is also dealt with. It is shown that the synovial fluids of rheumatic disease can be classified with greater sensitivity and of normal individuals with higher specificity.

Keywords : Fluorescence spectroscopy, synovial fluid, emission spectra, intensity ratio parameter.

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Photophysical properties of native fluorescence present, have been considered as a useful parameter to study alterations in the functional, morphological and micro-environmental changes in the cells and tissues. For example, the differences in the native fluorescence of collagen, elastin and more generally, protein due to the presence of aromatic amino acids like tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are related to the structural arrangement of cells and tissues [1–4]. A change in the state of the tissues, such as those occurring in physiological processes or in connection with the onset of a disease, results in modifications of the amount and distribution of the fluorophores. Many applications of native fluorescence have been reported, showing its non-invasive and sensitive approach both in the characterization and in the discrimination of normal tissues from pathological conditions of tissues [5]. Based on these facts, it is for the first time to the best of our knowledge, that a pilot study in the analysis of synovial fluid aspirated from rheumatic patients and healthy volunteers using native fluorescence spectroscopy, has been made. Synovial fluid is a viscous liquid that lubricates and nourishes healthy joints [6]. In many patients with arthritis, the composition of synovial fluid is altered and its viscosity reduced due to breakdown of many of the proteoglycan complexes, resulting in loss of lubrication and eventual damage to the joint.

Fluids were aspirated from the knee joints of patients suffering from rheumatic diseases and normal healthy volunteers. Out of 20 patients, 15 of them were males and 5 of them were females. Patients were adults of age at 17 to 65 years registered at Department of Rheumatology and Department of Orthopedics, Madras Medical College, Chennai, India, during April to October 2002. A volume of 200 μl of the sample was diluted with 6 mL of 0.9% NaCl solution. The diluted sample was mixed well with a vortex cyclo-mixer (Remi, India) and a volume of 3 ml was taken in the cleaned cuvette. The fluorescence emission spectra were recorded using a commercially available spectrofluorometer (ISA-SPEX Fluromax2, USA) at the Department of Physics, Medical Physics Section, Anna University, Chennai. Fluorescence measurements have been done on the synovial fluid by using 150W Xenon lamp source. The gratings in the

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excitation and emission spectrometers have a groove density of 1,200 grooves/mm and are blazed at 330 nm and 500 nm respectively. The entrance and exit ports of each spectrometer have continuously adjustable (in 0.025 mm increments) computer-controlled slits. The slits of the excitation spectrometer determine the amount of light passing through the excitation spectrometer to the sample. The emission spectrometer slits control the intensity of the fluorescence signal recorded by the signal photomultiplier detector. The excitation and emission slit widths were kept at 2 nm and 3 nm, respectively. The fluorescence emission characteristics of both normal and rheumatic synovial fluids can be analyzed at different excitation wavelengths such as 280 nm, 290 nm, 300 nm and 320 nm. In the present study, we confined our presentation of fluorescence emission characteristics at the wavelength of 280 nm and three methods have been applied to quantify the spectral data of fluorescence emission spectra and classify the normal from the diseased fluid. They are (i) emission intensity variation, (ii) the peak shift in the fluorescence emission wavelength of the normalized single peak spectra and (iii) the differences in the integrated intensity of the two spectra using the area under the curve analysis.

The representative fluorescence emission spectrum of normal and healthy synovial fluid at 280 nm excitation is shown in the Figure 1a. The corresponding excitation

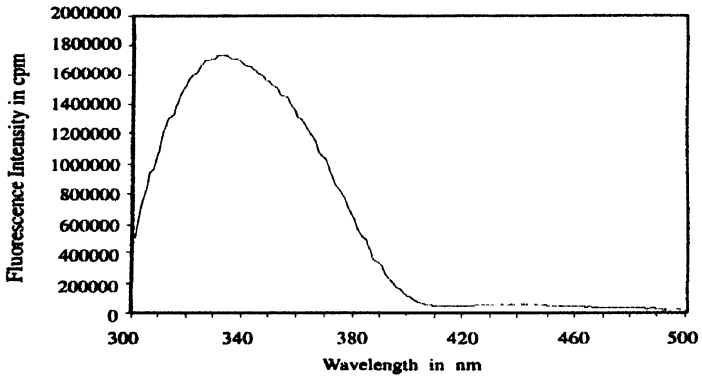

Figure 1a. Representative fluorescence emission spectrum of synovial fluid at 280 nm excitation.

spectra for 340 nm emissions are illustrated in the Figure1b. The spectra at 280 nm excitation consist of two bands. One located around 340 nm and the other around 440 nm. The amplitude of the band around 340 nm is stronger, narrower and more prominent than the secondary band around 440 nm. Studies based upon fluorescence spectra and laser-induced fluorescence spectra of cervical, oral tissues at normal and malignant conditions have

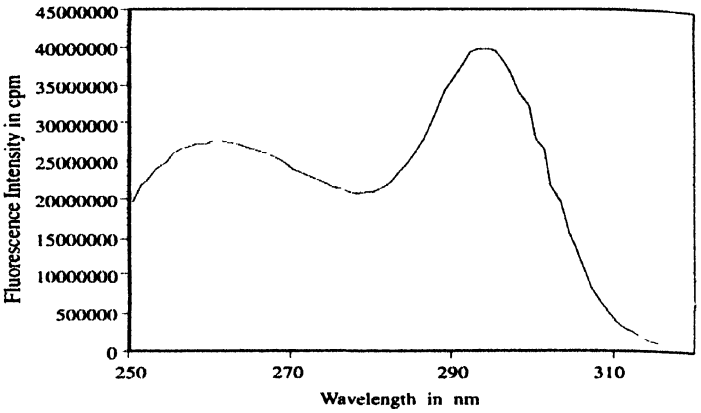

Figure 1b. Representative fluorescence excitation spectrum of synovial fluid at 340 nm emission.

already demonstrated that the peak at 340 nm is ascribed to tryptophan amino acid residues [7] and the intensity of the secondary band around 440 nm may be due to the presence of NADH, elastin and collagen [8,9]. The averaged fluorescence emission spectra of normal and diseased synovial fluid at 280 nm excitation are shown in the Figure 2. Considerable differences in the fluorescence

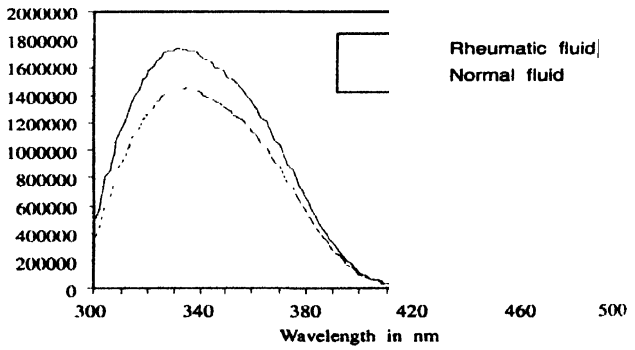

Figure 2. Averaged fluorescence emission spectra of normal and rheumatic synovial fluids at 280 nm excitation.

emission spectral profiles are perceived between the normal and diseased fluid. The averaged emission intensity around 334 nm for diseased fluid is lower than that of normal fluid. The intensity of the secondary peak around 440 nm is weak for normal fluid and also for diseased fluid. In some cases, it is not at all available. The normalized average fluorescence emission spectra of normal and diseased fluids are shown in Figure 3. It is observed that the primary band around 334 nm of the diseased fluid shows an average blue shift of 6 nm with respect to the primary band of the normal fluid.

Generally, it is reported that the native fluorescence of a protein is a concoction of the fluorescence from various aromatic amino acid residues, in particular, at an excitation

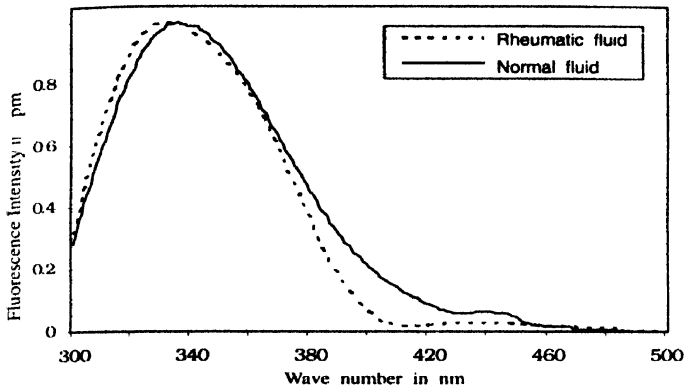

Figure 3. Normalized fluorescence emission spectra of normal and rheumatic synovial fluid at 280 nm.

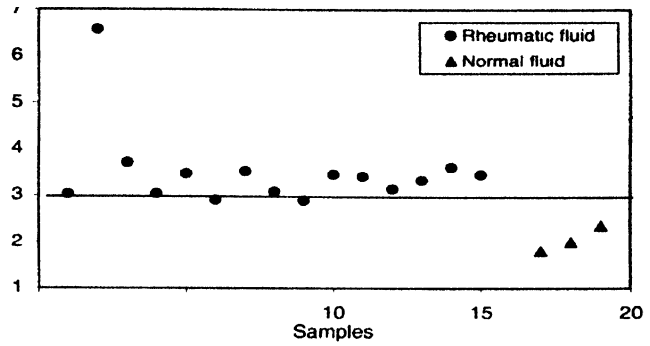

Figure 4. Distribution of intensity ratio parameter (IRP) values for normal and rheumatic fluids.

of 280 nm [1]. Most of the emissions are due to tryptophan residues with few emissions due to tyrosine and phenylalanine. Due to higher fluorescence quantum yield of tryptophan, resonance energy transfer from proximal phenylalanine to tyrosine and from tyrosine to tryptophan, the emission spectrum of tissues or fluids containing three residues usually resembles that of tryptophan [3]. Further, the photophysical characteristics of tryptophan amino acid is very much dependent on its surrounding micro environmental conditions, such as its solvent polarity, *pH* and viscosity. The fluorescence spectrum peak shifts to shorter wavelengths as the polarity of the solvents surrounding the tryptophan residues decrease. Further, it has been reported that the tryptophan residues which are buried in the hydrophobic core of proteins, can have a spectral blue shift of 10 to 20 nm when compared with exposed tryptophan. Hence, the blue shift of diseased fluid spectrum may be due to the presence of tryptophan residues under low solvent polarity environment and more buried conditions with respect to normal tissue protein or partial folding of proteins. The band around 440 nm is lesser for rheumatic fluid than those of normal indicating that the distribution and/or conformation of NADH, elastin and collagen may be altered when normal fluid converts into diseased state [10,11].

Although many intensity ratio parameters have been analyzed in the emission spectra, the IRP analysis is limited to wavelengths 323 nm and 393 nm *i.e.* $I_{(323)}/I_{(393)}$ as it shows better discrimination than other IRP values. Figure 4 shows the distribution of IRP values for normal and diseased fluid. The average values of the intensity ratio $I_{(323)}/I_{(393)}$ for normal and rheumatic synovial fluids are 1.791, 4.019, respectively. The mean value between them is 2.905 and it is used as a decisive factor to separate the rheumatic fluids from normal fluid. The critical ratio value 2.905 separates the diseased fluids from the normal with specificity, sensitivity and overall accuracy of 89.47%, 100% and 94.7% respectively. Sensitivity is defined as the fraction of diseased samples correctly classified and the specificity is defined as the fraction of the normal samples correctly classified. In clinical applications, the sensitivity and specificity are reported for the discrimination of diseased tissues from normal tissues. In the majority of clinical studies performed, the sensitivity and specificity are greater than 80%, reflecting the high classification accuracy of fluorescence spectroscopy. The sensitivity and specificity reported here are similar to clinical modalities that are used routinely.

The integrated intensity under the fluorescence emission spectra for normal and diseased fluids was also computed. Integrated intensity under the curve for the entire area of the spectra, are calculated by using trapezoidal rule,

$$\text{AUC} = h/2 \quad (y_1 + y_n) + 2\sum_{i=2}^{n-1} y_i$$

where h is the interval between the fluorescence intensity wavelengths, y_i denotes the absorption value at i-th wavelength. Figure 5 shows the distribution of the values of integrated intensity of area under the curve for the normal and rheumatic afflicted fluid. The average values of the area under the curve for normal and diseased fluids are 107.22 and 102.04, respectively. The mean value between them is 104.63 and it is used as a decisive factor to separate the diseased fluid from the normal fluid. The critical value 104.63 separates the diseased fluid from the normal with specificity, sensitivity and overall accuracy of 82%, 100% and 92% respectively.

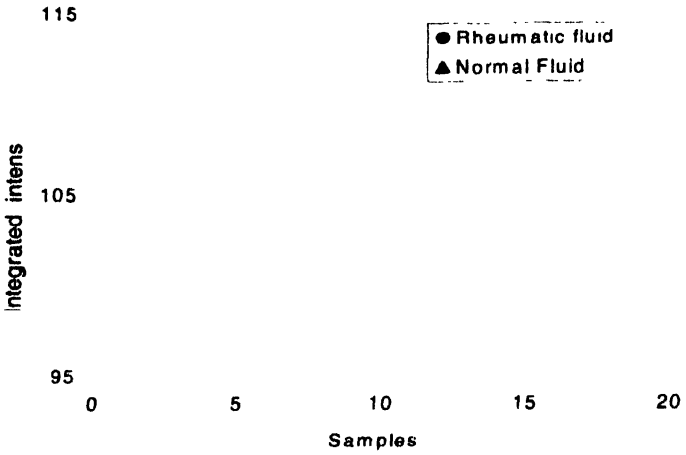

Figure 5. Distribution of integrated intensity of area under the curve for the normal and rheumatic fluids.

Native fluorescence emission spectra were studied to evaluate the potential of fluorescence spectroscopy to characterize the normal synovial fluid from the rheumatic synovial fluid. It is observed that the native fluorescence characteristics of fluorophores such as tyrosine, phenylalanine, tryptophan, elastin and NADH exhibit significant spectral differences between normal and diseased fluid, which may be attributed to the conformation changes and different micro-environments of these fluorophores in the synovial fluid. However, further studies are to be carried out with more number of samples to elucidate the reasons what could be the etiology for rheumatism and why the synovium is targeted.

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